Endoplasmic Reticulum Stress in Retinal Vascular Degeneration: Protective Role of Resveratrol

Chuanzhou Li,1 Leilei Wang,1 Kun Huang,2,3 and Ling Zheng1

PURPOSE. Endoplasmic reticulum (ER) stress has been demonstrated to contribute to neurodegeneration in multiple ocular diseases. However, whether ER stress can induce vascular degeneration in the retina remains unknown. We investigated the possible role of ER stress in retinal vascular degeneration in vivo, and the effects of resveratrol on tunicamycin and ischemia and reperfusion (I/R)-induced retinal vascular degeneration.

METHODS. Different dosages of tunicamycin, an ER stress inducer, were injected into the vitreous of mouse eyes. Retinal I/R injury was induced by elevating the intraocular pressure for 60 minutes followed by reperfusion in mice. Two dosages of resveratrol (5 and 25 mg/kg body weight per day) were administered 2 days before retinal I/R injury, while 100 μM resveratrol were injected into the vitreous together with tunicamycin. Formation of acellular capillaries was assessed 7 days after I/R injury and tunicamycin injection, while cell bodies in ganglion cell layer and brain-specific homeobox/POU domain protein 3A (Brn3a) staining on retinal flat-mounts were analyzed 4 days after I/R injury. ER stress markers, including eukaryotic initiation factor 2α (eIF2α), CCAAT enhancer-binding protein homologous protein (CHOP), immunoglobulin binding protein (Bip), inositol requiring enzyme 1 (IRE1α), (JNK)1/2, and Xbp1 splicing, were examined by RT-PCR, or Western blots or immunostaining from retinas 1 or 2 days after tunicamycin injection and I/R injury.

RESULTS. Tunicamycin caused ER stress and capillary degeneration in vivo, both of which were inhibited by resveratrol. Pretreatment of high dosage of resveratrol also significantly inhibited retinal I/R injury-induced capillary degeneration; however, neither of the dosages prevented the injury-induced neurodegeneration. Levels of CHOP, phosphorylated eIF2α, IRE1α, phosphorylated JNK1/2, Xbp1 splicing and Bip were elevated after I/R injury. High dosage of resveratrol pretreatment inhibited the injury-induced up-regulation of eIF2α-CHOP and IRE1α-XBP1 pathways.


Retinal ischemia is one of the causative factors in various ocular diseases, including acute close-angle glaucoma and diabetic retinopathy.1 Vascular and neuronal degeneration observed in these diseases are the key histological hall-markers that contribute to retinal dysfunction and vision loss in patients.2–5 Retinal ischemia and reperfusion (I/R) injury is a commonly used in vivo model to mimic neuronal and vascular degeneration.6–7 Through this model, the mechanisms underlying these degenerations and the therapies preventing the degenerations are studied.

One mechanism that contributes to the development of neuronal degeneration in the retina is endoplasmic reticulum (ER) stress. Upon accumulation of unfolded or misfolded proteins, ER activates three branches of adaptive responses to maintain cellular homeostasis and promote cell survival (see reviews8–10). However, when the protein folding ability cannot match the demand, ER stress is triggered and ER stress-dependent apoptotic pathway is evoked.10–11 Elevation of ER stress has been found in multiple animal models of retinal diseases, including retinitis pigmentosa,12 glaucoma,13,14 and diabetic retinopathy.15–17 The critical role of ER stress in neurodegeneration in the retina also is demonstrated. Retinal neurodegeneration, which was characterized by ganglion cell loss and reduction of retinal thickness, has been observed after intravitreal injection of ER stress inducer, such as tunicamycin.18,19 Mice that are deficient in CCAAT enhancer-binding protein homologous protein (CHOP), a transcription factor mediating ER stress-dependent cell death, are resistant to N-methyl-D-aspartic acid (NMDA)-induced retinal neurodegeneration.20 However, whether ER stress also contributes to vascular degeneration in the retina is not known yet.

Resveratrol (trans-3,5,4′-trihydroxystilbene), abundantly found in grape skins,21 is a natural polyphenolic phytoalexin with multiple bioactivities, including anti-oxidative22–25 and anti-inflammatory24,25 effects. The protective effects of resveratrol in several ocular diseases, including light/oxygen-induced retinopathy,26,27 endotoxin-induced uveitis,24 and diabetic retinopathy,28 have been demonstrated. However, the information on whether resveratrol could elicit protective effects on retinal I/R-induced neurovascular degeneration is lacking.

We investigated ER stress inducer tunicamycin-induced retinal vascular degeneration. The roles of resveratrol on tunicamycin or retinal I/R-induced ER stress and retinal vascular degeneration were evaluated. The results demonstrated that intravitreal injection of tunicamycin triggered ER stress in whole retina and retinal vasculature, and subsequent-induced capillary degeneration. Resveratrol inhibited tunicamycin and retinal I/R-induced capillary degeneration and overexpression of ER stress markers. Our findings uncovered a possible role of ER stress in retinal vascular degeneration, and
a new therapeutic role of resveratrol on retinal vascular dysfunction.

**MATERIALS AND METHODS**

**Animals**

C57BL/6 mice were obtained from Wuhan University Animal Laboratory, and housed in ventilated microisolator cages with free access to water and food. All procedures involving the animals conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and approved by the Committee on Ethics in the Care and Use of Laboratory Animals of Wuhan University.

**Retinal I/R Model and Resveratrol Administration**

Retinal I/R was induced as previously reported. Briefly, after pupil dilation, the anterior chamber of one eye in the mouse was cannulated with a 35-gauge needle connected to a reservoir containing 0.9% NaCl. Pressure in the eye was increased to 80–90 mmHg with a pressure infuser (Infus Surg; Ethox Corp., Buffalo, NY). The duration of ischemia was 60 minutes. Retinal ischemia was confirmed by whitening of the iris and the fundus under microscopic observation. After ischemia, the needle was withdrawn and retinal circulation was reflowed. The other non-injured eye of the same animal served as the control. A sham operation without withdrawal and retinal circulation was reflowed. The other non-injured retinal sections of any experimental groups. The nuclei in GCL (not including nuclei in the vessels) were counted. The result was reported as number of nuclei in GCL per 250 μm of retinal surface as reported previously.

**Brain-Specific Homeobox/POU Domain Protein 3A (Brn3a) Immunofluorescence Staining**

Four days after retinal I/R injury, retinal flat-mounts were freshly isolated. After fixation in 4% formalin at room temperature, flat-mounts were washed and treated with 2% Triton X-100 for 15 minutes, then the immunostaining was performed using MOM kit (Vector Laboratory, Burlingame, CA) with anti-Brn3a (1:100 dilution; Millipore, Temecula, CA) and Alexa Fluor 488 labeled anti-mouse IgG (1:300 dilution; Invitrogen, Carlsbad, CA). After extensive washing, anti-fading mounting solution containing DAPI (Beyotime Biotech) was added to the samples. Images were taken under confocal microscope (Olympus, FV1000) at multiple magnifications (40×, 100×, and 600×). Brn3a+ cells in 20–30 different areas (600× magnification) of each retina were counted and reported per square millimeter of retinal area.

**RNA Isolation and RT-PCR**

Total RNA was isolated from retina using RNeasy Plus (TaKaRa Biotechnology, Dalian, China). Total RNA (1 μg) was reverse transcribed into cDNA using M-MuLV first strand synthesis system (Invitrogen). The absence of specific gene transcripts was assessed by PCR. The primer sequences were listed in supplementary Table S1 (http://www.iovs.orglookup/suppl/doi:10.1167/iovs.11-8406/-/DCSupplemental). PCR products were separated by electrophoresis using 1.5% agarose gels, and images were photographed by Molecular Imager Gel Doc XR (Bio-Rad, Hercules, CA). Band density was quantified using Quantity One 1-D Analysis Software (Bio-Rad). mRNA levels were quantitated first against 18S rRNA levels in the same sample, then normalized to non-injured group, which was set up as 1-fold, with the exception that Xhp1 splicing was reported as the ratio of spliced Xhp1 (Xhp1s)/total Xhp1 (Xhp1t).

**Western Blot Analysis**

Retinas were isolated and sonicated in RIPA buffer (Beyotime Biotech, China). A total of 20–40 μg proteins per sample were separated by SDS-PAGE and electroblotted onto PVDF membrane (Millipore). After blocking, antibodies for p-eIF2α (CST, Danvers, MA), total-eIF2α (CST), CHOP (Epitomics, Burlingame, CA), Immunoglobulin binding protein (Bip; BD Transduction Laboratories, Franklin Lakes, NJ), inositol requiring enzyme 1α (IRE1α; CST), phospho-C-Jun N-terminal kinase (JNK)1/2 (CST), total-JNK1/2 (CST), and Jαtin (Sigma, St. Louis, MO) were applied overnight at 4°C. All blots were washed and incubated with respective secondary antibody (Bio-Rad). Protein bands detected by the antibodies were visualized by ECL reagent (Millipore) following exposure on X-Omat film. Protein expression levels were quantified.
by the Quantity One 1-D Analysis Software (Bio-Rad) as we reported previously.\textsuperscript{4,7} The protein levels, except for the phosphorylated proteins, first were quantitated relative to β-actin in the same sample, and then the relative protein expression levels in different groups were normalized to the non-injured group, which set up as 1-fold. The levels of the phosphorylated proteins were quantitated first relative to their corresponding total protein levels in the same sample, and then the relative phosphorylated protein expression levels in different groups were normalized to the non-injured group, which was set up as 1-fold.

**Statistical Analysis**

All results were expressed as the mean ± SD. Data were analyzed by the nonparametric Kruskal-Wallis test followed by the Mann-Whitney U test. Differences were considered statistically significant when \( P < 0.05 \). The changes were considered as trends when \( 0.05 < P \leq 0.08 \) also were reported.

**RESULTS**

**Resveratrol Inhibits Tunicamycin-Induced Vascular Degeneration in the Retina**

Morphology of the vascular tree for the vehicle-injected retinas was as normal as that for the non-injected retinas (data not shown). However, seven days after tunicamycin injection, there was a dramatic increase in formation of completed degenerated capillaries (acellular capillaries), and ongoing degenerating capillaries (condensed and fragmental-like nuclei within capillary tubes) in the tunicamycin-injected retinas (Fig. 1A). In retinas injected with 0.1, 1, and 1.5 μg tunicamycin, there were 32.7 ± 11.9, 121.8 ± 17.7, and 190.0 ± 34.9 acellular capillaries per mm² retina, compared to 3.0 ± 0.4 acellular capillaries per mm² in the vehicle injected retinas (\( P = 0.01 \), Fig. 1B). Moreover, 100 μM resveratrol significantly inhibited the formation of acellular capillary induced by 0.1 μg tunicamycin (\( P < 0.01 \)). No significant differences in the formation of acellular capillaries were found between vehicle-injected retinas and non-injected retinas (Fig. 1B).

**Resveratrol Inhibits Retinal I/R-Induced Vascular Degeneration**

Morphology of acellular capillaries after tunicamycin injection was similar to the capillary degeneration found after retinal I/R injury as we reported previously.\textsuperscript{4,7} Since resveratrol has been reported to have vascular protective effects on retinal neovascularization,\textsuperscript{29} the possible effect of resveratrol in vascular degeneration after retinal I/R injury was investigated. Seven days after injury, there was a significant increase in the formation of acellular capillaries in I/R-injured retinas compared to that in non-injured retinas (Fig. 2, \( P < 0.01 \)), while there was no significant difference in the number of degenerated capillaries between non-injured and sham operated retinas (Fig. S1C, http://www iovs org/lookup/ suppl/doi:10.1167/iovs.11-8406/-/DCSupplemental). Resveratrol administration (25 mg/kg BW/day) significantly inhibited I/R injury-induced acellular capillary formation compared to I/R-injured groups (\( P < 0.01 \)). However, low dosage resveratrol administration (5 mg/kg BW/day) showed no effects on I/R injury-induced acellular capillary formation.

**Resveratrol Does Not Prevent Retinal I/R-Induced Neuronal Cell Loss**

Neuronal degeneration is another I/R-induced pathological change besides capillary degeneration in the retina. Effects of resveratrol on retinal I/R-induced neuronal cell loss were evaluated by two different methods. Consistent with our previous observations,\textsuperscript{4,7} four days after retinal I/R injury there was a significant cell loss in GCL in I/R-injured retinas compared to that in non-injured retinas (\( P < 0.01 \), Figs. 3A, 3B), while there was a similar cell number in GCL between non-injured and sham operated retinas (Fig. S1D, http://www. iovs org/lookup/ suppl/doi:10.1167/iovs.11-8406/-/DCSupplemental). However, pre-treatment of neither high nor
low dosage of resveratrol significantly inhibited I/R-induced cell loss in GCL (Figs. 3A, 3B).

To confirm the effect of resveratrol on retinal I/R-induced neurodegeneration, a specific retinal ganglion cell marker was used. Brn3a is a transcription factor expressed specifically in the nuclei of ganglion cells. Representative images of Brn3a immunostaining of each group on retinal flat-mounts with different magnifications were shown in Figure 3C (40× and 100×) and Figure S2 (600×, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8406/-/DCSupplemental). Quantitative results demonstrated that the number of Brn3a+ cells declined from 1914 ± 196 cells in the non-injured group to 652 ± 269 cells in the I/R-injured group per mm² retinal area (P < 0.01, Fig. 3D). Consistent with the result of cell number counting in GCL, high dosage of resveratrol did not rescue I/R-induced ganglion cell loss (702 ± 187 Brn3a+ cells per mm² retinal area, Fig. 3D).

**Resveratrol Inhibits Retinal I/R-Induced Up-Regulation of Eukaryotic Translational Initiation Factor 2α (eIF2α)-CHOP Branch of ER Stress**

Effects of resveratrol on ER stress were investigated further after retinal I/R injury. Prolonged activation of ER stress results in phosphorylation of eIF2α, which leads to induction of CHOP, which initiates the ER stress-dependent apoptosis. The mRNA and protein levels of CHOP were examined three hours after the injury (Figs. S3A, S3B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8406/-/DCSupplemental). However, the transcript level of Chop was increased mildly one day after retinal I/R injury (P = 0.08, Figs. 4A, 4B). In addition to mRNA level, protein level of CHOP was 8-fold increased one day after retinal I/R injury (P < 0.05, Fig. 4B). Furthermore, p-eIF2α, the upstream factor that induces CHOP elevation, also was increased significantly 5.3-fold one day after I/R injury (P < 0.05, Fig. 4B). All these changes were inhibited significantly by high dosage of resveratrol pretreatment (P < 0.05, Figs. 4A, 4B). There was no difference in the total eIF2α level among the experimental groups.

To clarify whether resveratrol affects ER stress on vascular part of the retina, CHOP expression on retinal vasculatures of different experimental groups was evaluated. We observed elevated CHOP expression in the nuclei of vascular cells one day after I/R injury, and this injury-induced vascular CHOP overexpression was inhibited significantly by the pretreatment high dosage of resveratrol (Fig. 4C).

**Resveratrol Inhibits Retinal I/R-Induced IRE1α Up-Regulation and Xbp1 Splicing**

IER1α, another ER stress sensor localized on ER membrane, also was analyzed. Although its level was not changed three hours after the injury (Figs. S3A, S3B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8406/-/DCSupplemental), an 8.1-fold
up-regulation of IER1α was triggered one day after I/R injury ($P < 0.05$). Pretreatment of high dosage of resveratrol inhibited significantly the injury-induced IRE1α overexpression ($P < 0.05$; Figs. 5A, 5B). No difference was found in the total JNK1/2 levels among the experimental groups one day after the injury. However, one day after retinal I/R injury, a 3.6-fold increase in phosphorylation level of JNK1/2 was observed ($P < 0.05$), while resveratrol treatment did not inhibit this alternation.

Another possible downstream target of IRE1α activation, Xbp1 splicing, was investigated. Xbp1 splicing, shown as the ratio of Xbp1s/Xbp1II, was increased significantly one day after I/R injury ($P < 0.05$, Figs. 5C, 5D). Pretreatment of high dosage of resveratrol inhibited significantly the I/R injury-induced Xbp1 splicing ($P < 0.05$, Figs. 5C, 5D).

**Resveratrol Inhibits Retinal I/R-Induced Overexpression of Bip**

Bip is a molecular chaperone that modulates ER homeostasis and helps protein refolding under stressed conditions. Its protein level was increased mildly three hours after I/R ($P = 0.08$; Figs. S3A, S3B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8406/-/DCSupplemental), the mRNA and protein levels of Bip also were elevated one day after the injury ($P < 0.05$ and $P = 0.08$, respectively, Figs. 6A, 6B), and pretreatment of high dosage resveratrol inhibited the injury-induced increase in Bip mRNA level ($P < 0.05$) but not the protein level.

**Resveratrol Inhibits Tunicamycin-Induced ER Stress in the Retina**

ER stress markers also were investigated in the retinas after tunicamycin injection. RT-PCR results showed significantly increased mRNA levels of ER stress markers, including Chop, growth arrest and DNA damage-inducible protein 54 (Gadd34), Bip, heat shock protein 70 (Hsp70) and protein disulfide isomerase (Pdi), in tunicamycin injected retinas (Fig. S4A, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8406/-/DCSupplemental). Moreover, CHOP expression was up-regulated in retinal vasculatures after tunicamycin injection (Fig. S4B, http://www.iovs.org/lookup/suppl/doi:10.1167/
Western blots demonstrated that 0.1 g tunicamycin injection triggered significant overexpression of ER stress markers (CHOP, IRE1α, Bip) compared to vehicle-injected retinas, and these increases were inhibited significantly by resveratrol treatment (Fig. 7).

**DISCUSSION**

Neuronal and vascular cells are two major targets for retinal damages in various ocular diseases. Neuronal and vascular degeneration has been observed in retinal I/R injury models. Several mechanisms through which retinal I/R injury contributes to neurodegeneration have been demonstrated. However, investigations on the underlying mechanisms of I/R-induced vascular degeneration are lacking. In our study, we demonstrated that tunicamycin injection and retinal I/R injury could induce prolonged ER stress and acellular capillary formation in vivo, and these alternations can be inhibited dramatically by administration of resveratrol, suggesting ER stress might be one contributor to vascular dysfunction after retinal injury.

**FIGURE 5.** Resveratrol inhibits retinal I/R injury-induced IRE1α-XBP1 up-regulation. (A) Representative Western blots of IRE1α, p-JNK1/2, and total JNK1/2 of different experimental groups. (B) Densitometric quantitative results of IRE1alpha and JNK1/2 activation. (C) Representative images of mRNA levels of Xbp1s, Xbp1t, and 18S rRNA of different experimental groups. (D) Densitometric quantitative results of Xbp1 splicing. n = 4–5 per group; *P < 0.05 compared with non-injured retinas, *P < 0.05 compared with I/R-injured retinas.

**FIGURE 6.** Resveratrol inhibits retinal I/R-induced increase of Bip transcription. (A) Representative images of mRNA levels of Bip and 18S rRNA (upper panel) and the quantitative results (bottom panel) of different experimental groups. (B) Representative Western blots of Bip and β-actin (upper panel), and the densitometric quantitative results (bottom panel) of different experimental groups. n = 4–5 per group; *P = 0.08, compared with non-injured retinas; *P < 0.05, compared with non-injured retinas; *P < 0.05, compared with I/R-injured retinas.
In the endotoxin-induced uveitis model, splicing and IRE1 (Figs. 4, 5, 7). Moreover, retinal I/R injury also causes an increase in ER stress-related markers, including CHOP and Bip expression levels, and retinal cell death. Furthermore, retinal detachment induces significant increases in CHOP and Bip expression levels, and retinal cell death. In addition, I/R injury induced by central retinal artery occlusion also causes an increase in ER stress-related markers, including IRE1α and phosphorylated JNK1/2 in GCL. Activation of ER stress not only is associated with retinal diseases, but also can be a causative factor in the development of neurodegeneration in the retina, since intravitreal injection of ER stress inducer, such as tunicamycin, induces rapid retinal neurodegeneration. Bip induces the CHOP overexpression and, thus, attenuates neurodegeneration in retina injuries induced by NMDA and tunicamycin. Consistent with this, NMDA-induced neurodegeneration is reduced in the mice lacking Bip. Activation of ER stress also was found in our study using a high ocular pressure-induced I/R injury model, as demonstrated by the significant increase in CHOP p-eIF2α, Bip, Xbp1 splicing and IRE1α overexpression and JNK1/2 activation (Figs. 4–6).

The rationale for using resveratrol in our study is due to its vascular protective effects, which have been studied in some ocular diseases. In the endotoxin-induced uveitis model, resveratrol administration leads to suppression of leukocyte adhesion on the retinal vasculature by its anti-oxidative and anti-inflammatory effects in a dose-dependent manner. Oral administration of resveratrol also inhibits the retinal neovascularization via inhibition of vascular endothelial growth factor in Vldlr−/− mice. The mice with very low-density lipoprotein receptor (VLDLR) mutant that have pathologic features similar to macular telangiectasia. In our study, resveratrol attenuates tunicamycin and I/R injury-induced vascular degeneration (Figs. 1, 2), and a direct effect of resveratrol on I/R-induced vascular CHOP overexpression also is demonstrated (Fig. 4C), which is consistent with the vascular protective roles of resveratrol.

The effects of resveratrol on ER stress are controversial based on several in vitro studies. After 24 hours of incubation, treatments with 10–100 μM resveratrol have been shown to cause apoptosis, and induce overexpression of CHOP and Bip in cancer cell lines. In contrast, other investigators have demonstrated that in mouse embryonic fibroblasts co-treated with tunicamycin for 6 hours, treatment with 10 μM resveratrol inhibits significantly tunicamycin-induced CHOP and Bip overexpression. However, to our knowledge, the effects of resveratrol on ER stress in rodent models were unclear. In our study, resveratrol inhibited significantly tunicamycin and retinal I/R injury-induced up-regulation of ER stress markers, including CHOP and IRE1α (Figs. 4, 5, 7). Moreover, retinal I/R and tunicamycin-induced CHOP up-regulation in vascular cells as well as capillary degeneration were blocked by resveratrol treatment, suggesting ER stress might be a direct link between I/R injury and the following capillary degeneration. Therefore, resveratrol probably protects vasculature from degeneration via attenuating ER stress pathways on retinal vessels after I/R injury.

Resveratrol has been reported to have retinal neuroprotective effects. Resveratrol pretreatment at a dosage of 50 mg/kg BW for 5 days prevents light-induced retinal neurodegeneration. Treating the diabetic mice with resveratrol at a dosage of 20 mg/kg BW for 4 weeks inhibits neuronal apoptosis in the retina via stimulating the activity of Ca2+/calmodulin-dependent protein kinase II. However, in our study, neither 5 nor 25 mg/kg BW for day of resveratrol showed protective effects on retinal I/R, as indicated by two different methods (Fig. 3). This difference may be due to, but not limited to, the different dosages of resveratrol and/or the different durations of resveratrol exposure that were used. Under our experimental conditions, the fact that resveratrol inhibits the cell death of retinal capillary cells but not the neuronal cells, at least, suggests vascular and neuronal elements in the retina may be targeted differently by resveratrol.

### Figure 7
Resveratrol inhibits tunicamycin-induced increase in ER stress markers. Representative Western blots of CHOP, IRE1α, Bip, and β-actin of different experimental groups were shown.

### Figure 8
Possible mechanism of the protective effects of resveratrol on retinal I/R injury and tunicamycin-induced vascular degeneration. 0, inhibition by resveratrol.
In summary, our study suggested that ER stress contributes to retinal capillary degeneration in vivo. We demonstrated that retinal I/R injury and tunicamycin trigger ER stress activation and subsequent vascular degeneration. Resveratrol suppresses tunicamycin and I/R injury-induced ER stress via inhibition of expressions of CHOP and IRE1α, thus resulting in protection against the injury-induced capillary degeneration (Fig. 8). Our study revealed a possible role of ER stress in the development of capillary degeneration, and suggested that resveratrol could be a potential drug candidate for vascular dysfunction in the retina.

Acknowledgments

The Research Core Facility of College of Life Sciences, Wuhan University, provided analytical supports.

References


